The Combination of MLN2238 with Interferon-alfa Results in Enhanced Cell Death in Melanoma

A Senior Honors Thesis

Presented in Fulfillment of the Requirements for Graduation *with Honors Research Distinction in Microbiology* in the Undergraduate Colleges of The Ohio State University

By

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Abstract

The proteasome plays an important role in the ordered degradation of cell components required for cell cycle progression in malignant and normal cells. Bortezomib is a proteasome inhibitor that has *in-vitro* activity against a variety of tumor cell types. In this project, a second-generation proteasome inhibitor MLN2238 was examined. It was hypothesized that MLN2238 would induce apoptosis in human melanoma cells. The melanoma cell lines A375 and WM1366 were treated with various concentrations of MLN2238 alone or in combination with interferon-alpha (IFN-alfa) – an FDA-approved immune boosting agent that has activity in melanoma. Alone MLN2238 induced apoptosis at 48 hours with an IC50 of approximately 35 nM. Apoptosis was first observed at 12 hours and was maximal at 48 hours. MLN2238-induced apoptosis was also associated with processing of the pro-apoptotic proteins caspase-3, -7, -8, and-9, and cleavage of PARP. The addition of IFN-α alone at a dose of $5x10^3$ U/mL to cell cultures had little effect on cell viability, but in combination with MLN2238 at 35 nM (IC_{50} dose) there was synergistic induction of apoptosis and cell death with 78.9 ± 4% cell death in the A375 (BRAF mutant) melanoma cell line. There was a smaller increase in the level of apoptosis with the combination treatment of MLN2238 and IFN-alfa in the BRAF WT (WM1366) cell line. The combination of IFN-alfa and 35 nM MLN2238 resulted in synergistic inhibition of cell proliferation as measured by the MTT assay in the BRAF mutant (A375) cell line. This combination treatment inhibited proliferation in BRAF WT cell lines (WM1366 and MeWo). These results imply that MLN2238 in combination with IFN-alfa may have efficacy in the treatment of advanced melanoma, especially in BRAF V600E melanoma.

Introduction

Melanoma is the deadliest skin cancer, accounting for 79% of skin cancer deaths [1,2,3]. In 2013, it was estimated that there will be 73,870 new cases of melanoma in the United States and 9,480 deaths from the disease [4]. Patient outcomes are largely dependent on the stage of disease at initial presentation. It is estimated that 82-85% of melanoma patients present with localized disease, which can often be effectively treated with surgery alone [5]. However, long-term survival in patients with metastatic disease remains poor. Traditional chemotherapeutic regimens including dacarbazine, temozolomide, high-dose interleukin-2 (IL-2), and paclitaxel with or without cisplatin or carboplatin have demonstrated only modest response rates (<25%) [5]. Recently, novel therapies including ipilimumab (a monoclonal antibody directed against cytotoxic T lymphocyte antigen-4) and vemurafenib (a specific inhibitor of signaling by mutated BRAF) have received FDA-approval for the treatment of metastatic melanoma. However, both agents possess unique limitations. Phase III trials involving ipilimumab revealed potential for serious autoimmune toxicity, with immune-related events occurring in 60% of patients [6]. Moreover, the overall response rate remains low (<25%), but is useful in a subset of patients that experience long term benefits [6]. Vemurafenib has shown improved response rates (48%) but is limited to patients with a V600 mutated BRAF gene [7]. In addition, the median duration of response is only 6 months [8]. These regimens highlight the need for new therapies with a better side effect profile.

The ubiquitin-proteasome signaling (UPS) pathway plays a critical role in the ordered, temporal degradation of transcription factors, cyclins, and cyclin-dependent

kinase inhibitors required for cell cycle progression [9]. Dysregulation in the UPS pathway is linked to the pathogenesis of various human diseases and therefore targeting components of UPS represents a novel therapeutic treatment strategy in the disease setting. Proteasome inhibition results in the stabilization and accumulation of cell regulatory proteins, leading to the activation of anti-proliferative signals, cell cycle disruption, activation of apoptotic pathways, and, ultimately, cell death [10,11].

Bortezomib is an antitumor compound that can selectively inhibit the 26S subunit of the proteasome in malignant cells [12]. Bortezomib was the first FDA approved proteasome inhibitor when it was approved in 2005. Single agent use of bortezomib has shown an effect in multiple myeloma and mantle cell lymphoma [12]. Phase II trials of single agent use in melanoma have not shown a response [13]. Several studies have evaluated the efficacy of bortezomib in combination with chemotherapy for the treatment of malignant melanoma. In general bortezomib has not shown clinical activity, but this may be due to the half life of this IV administered drug [13]. There is evidence that bortezomib administration increases IFN response proteins such as the phosphorylated form of STAT1 which demonstrates a potential relationship between the two agents [13].

IFN-alfa has been shown to induce apoptosis in some cancer cells and is able to sensitize other cancer cells to apoptosis [14]. While the mechanism of apoptotic resistance in melanomas is not completely understood, a role for Bcl-2, Mcl-1, and Fas has been described [10]. Our group has shown that bortezomib and IFN-alfa act synergistically to induce apoptosis in melanoma cell lines by activation of caspase-8 through the association of Fas and FADD. The combination of these agents was even

effective at inducing apoptosis in cells that over-expressed the pro-survival proteins Bcl-2 and Mcl-1 [14]. Combination treatment also led to increased survival and inhibited tumor growth in a murine tumor model [10]. Dr. Carson's group has also shown that bortezomib enhances the direct cytotoxic effect of IFN-alfa on melanoma cells through the induction of IFN-α response genes and increased phosphorylation of STAT1 [15]. This preclinical work led to the formation of an NCI-sponsored phase I clinical trial of bortezomib and interferon-alfa-2B in metastatic melanoma. Of the 16 patients accrued for the study, one patient (6.3%) experienced a partial response and seven patients (43.8%) exhibited stable disease. Progression free survival and overall survival were 2.5 months and 10.3 months, respectively [13]. The phase I clinical trial of bortezomib with IFN-alfa did not demonstrate potential activity possibly because of decreased serum concentrations due to IV administration of the proteasome inhibitor, which warrants the study of an oral proteasome inhibitor where steady drug levels can be achieved.

MLN9708 is a selective, orally bioavailable, novel second-generation proteasome inhibitor that specifically and reversibly inhibits the 20S proteasome [16]. MLN9708 rapidly hydrolyzes to its biologically active form (MLN2238) following exposure to aqueous solution or plasma [17]. Preclinical studies have demonstrated a shorter proteasome dissociation half-life as well as improved pharmacokinetics, pharmacodynamics, and antitumor activity in xenograft models as compared to bortezomib [16]. In the present study, we examined the antitumor activity of single-agent MLN2238 and MLN2238 drug combinations using *in vitro* malignant melanoma models. We hypothesized that the treatment of human melanoma cells with

MLN2238 and IFN-alfa drug combinations would result in enhanced tumor cell apoptosis.

Materials and Methods

Materials: The A375 human melanoma cell line was purchased from the American Type Culture Collection (ATCC Manassas, Virginia). The WM1366 and MeWo cell lines were obtained from Dr. Saldano Ferrone now of the Massachusetts General Hospital. MLN2238 (MLN9708) and bortezomib (Velcade, PS-341) were obtained from Millennium Pharmaceuticals, Inc. (Cambridge, MA). Recombinant human IFN-alfa was obtained from Schering-Plough, Inc (Kenilworth, NJ).

Annexin V-propidium iodine staining: Apoptosis-induced phosphatidylserine exposure and cell membrane integrity was measured in tumor cells by flow cytometric analysis using APC-Annexin V and PE-propidium iodide (PI; BD Pharmingen, San Jose, CA), as previously described [18]. Each analysis was performed using 10,000 – 30,000 events and provides the percentage of the cell population that is viable or apoptotic [18]. The externalization of phosphatidylserine (PS) residues on the cell membrane occurs first during apoptosis. Annexin V has a high affinity for PS and monitors it once PS transfers from the inner membrane to the outer membrane. Propidium iodide is not able to penetrate viable cell membranes. Once the integrity of the cell membrane begins to degrade during apoptosis, propidium iodide can then penetrate the cell. The combination of these two agents together provides a useful assay to determine whether a cell population is viable, apoptotic, or necrotic [18]. The full method is described in Appendix A.

Light microscopy. Differential interference contrast (DIC) images were obtained using an Olympus Fluoview 1000MPE confocal microscope equipped with a

LUMPlanFI/IR 40XW objective (N.A. 0.8) at 4 times optical zoom. All presented images were processed identically using Olympus Fluoview (v.2.1b) software.

Immunoblot analysis: Immunoblots were prepared to probe with antibodies specific for caspase-3, caspase-7, caspase-8, caspase-9, cleaved caspase-7, poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology, Danvers, MA), or β-actin (Sigma, St. Louis, MO). Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, immune complexes were detected using an enhanced chemiluminescence detection kit (Thermo Scientific, Waltham, MA).

Proliferation assays: The proliferation of melanoma cells treated with MLN2238 with or without IFN-alfa will be measured as absorbance at 570 nm using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Cell Proliferation Assay according to the procedure as previously described with modification [19]. The full method is described in Appendix B.

Results

Treatment of BRAF V600E mutant human melanoma cell lines with MLN2238 led to apoptotic cell death. The ability of MLN2238 to induce apoptosis of A375 (BRAF V600E mutant) and WM1366 (BRAF wild type) human melanoma tumor cells was evaluated via Annexin V/PI staining. Time course experiments were conducted using the A375 melanoma cell line and 35 nM dose of MLN2238. Apoptosis was measured by the Annexin/PI assay at 12, 24, and 48 hours and revealed that apoptosis began at 12 hours and increased until approximately 48 hours (Figure 1). 48 hours was determined to be optimal for the remaining experiments. Therefore, human melanoma tumor cells were treated for 48 hours with either complete medium alone or varying doses of MLN2238. As can be seen in Figure 1A, A375 cells treated with DMSO (solvent control) exhibited 1.73% cell death at the 48-hour time point. In comparison, the addition of 35 nM MLN2238 resulted in 58.7% cell death. The IC50 dose of MLN2238 was determined through a titration curve performed in duplicate (IC50 ≈ 32.1 nM; Figure 2B). Microscopic images of the A375 human melanoma cell line following 48-hour treatment with 65 nM MLN 2238 showed further evidence of apoptotic cell death (blebs, reduced cell volume) as compared to untreated cells (Fig. 2C).

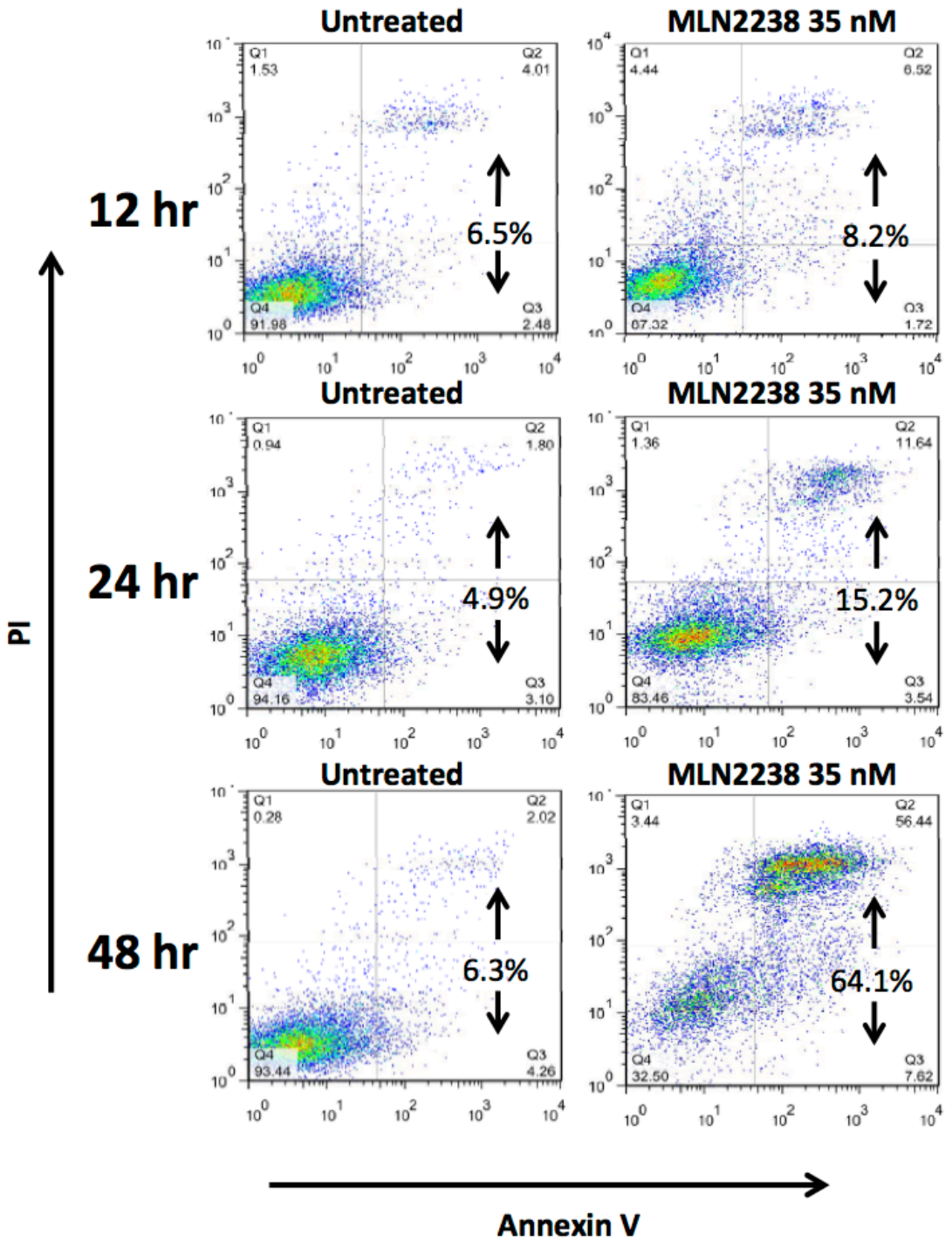


Figure 1. MLN2238 induces maximal apoptosis with 48-hour treatment in the BRAF mutant (A375) cell line. A375 BRAF mutant (V600E) human melanoma cells were plated on a 6 well plate at a density of 66,000 cells/mL and were treated with either medium alone with DMSO control (untreated) or 35nM MLN2238. Cells were incubated for 12 hours, 24 hours, or 48 hours. After incubation, the cells were subjected to an Annexin V/Propidium Iodide assay to determine the levels of apoptosis. The results indicated that apoptosis began at approximately 12 hours and increased until 48 hours.

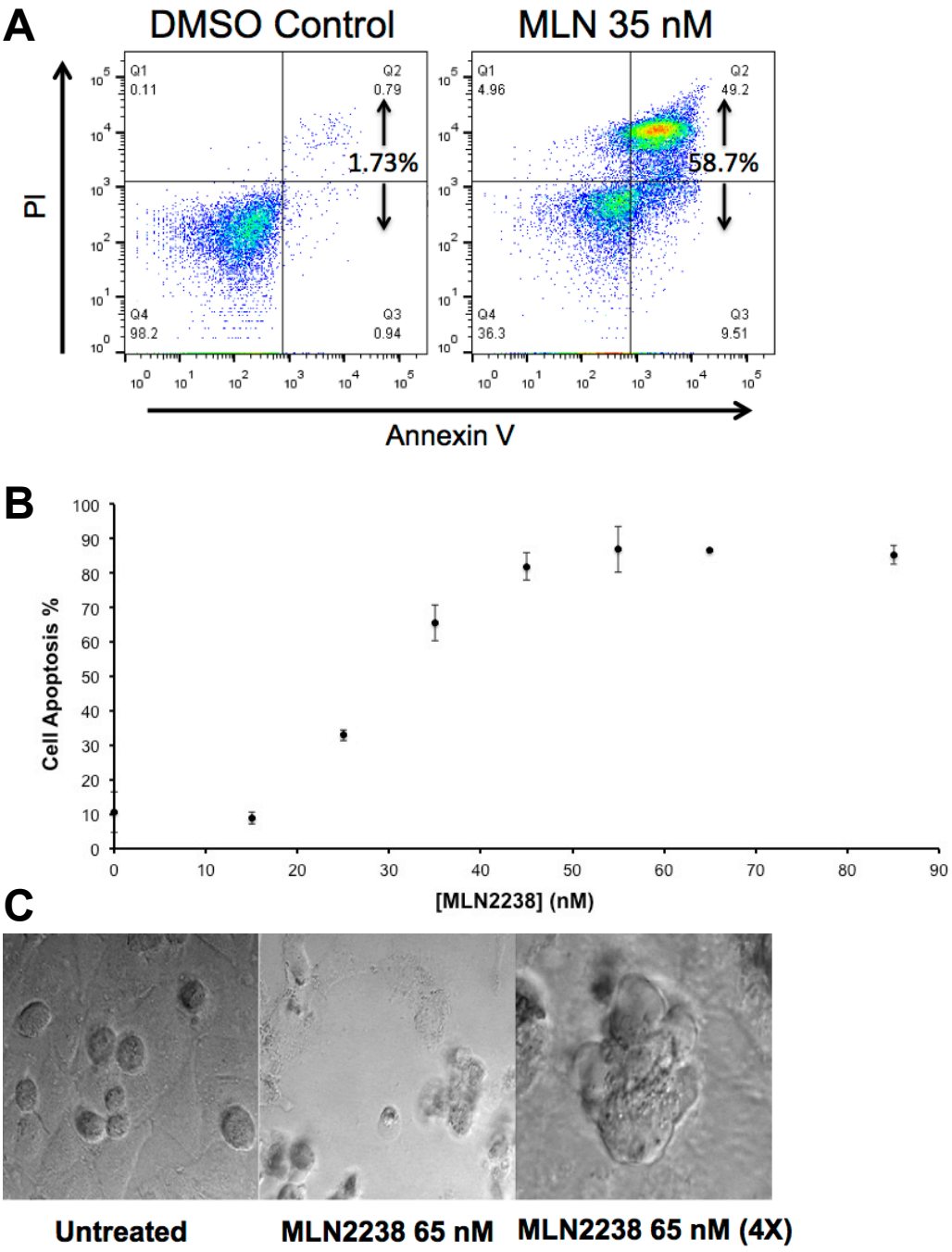


Figure 2. Treatment of BRAF V600E mutant human melanoma cell lines with MLN2238 led to apoptotic cell death. A375 BRAF mutant (V600E) human melanoma cells were plated on a 6 well plate at a density of 7 x 10^4 cells/mL and were treated for 48 hours with either medium alone or varying doses of MLN2238. After incubation, the cells were subjected to an Annexin V/Propidium Iodide assay to determine the levels of apoptosis. **A)** An example of the Annexin/PI assay for 35 nM MLN2238 is shown following 48-hour treatment of A375 cells. **B)** A titration was performed to determine the IC_{50} concentration. The MLN2238 IC_{50} value for the A375 cell line is approximately 32.1 nM. **C)** A375 BRAF mutant (V600E) human melanoma cells were treated for 48 hours with either cell culture media with DMSO (untreated) or 65 nM MLN2238. Microscopic images were taken after the 48-hour incubation. The images provided evidence of apoptotic cell death (blebs of reduced volume) as compared to untreated cells.

Treatment of the BRAF wild type human melanoma cell line with MLN2238 led to apoptotic cell death. A BRAF wild-type cell line (WM1366) was treated with MLN2238 for 48 hours (Figure 3). At the 48 hour time point, DMSO (solvent control) treated WM1366 cells displayed 5.43% cell death which was comparable to cells without DMSO present (Figure 3A). The dose at which MLN2238 caused 50% cell death was determined through the formation of a titration curve as was performed in the A375 cell line (approximately 38.4 nM; Figure 3B).

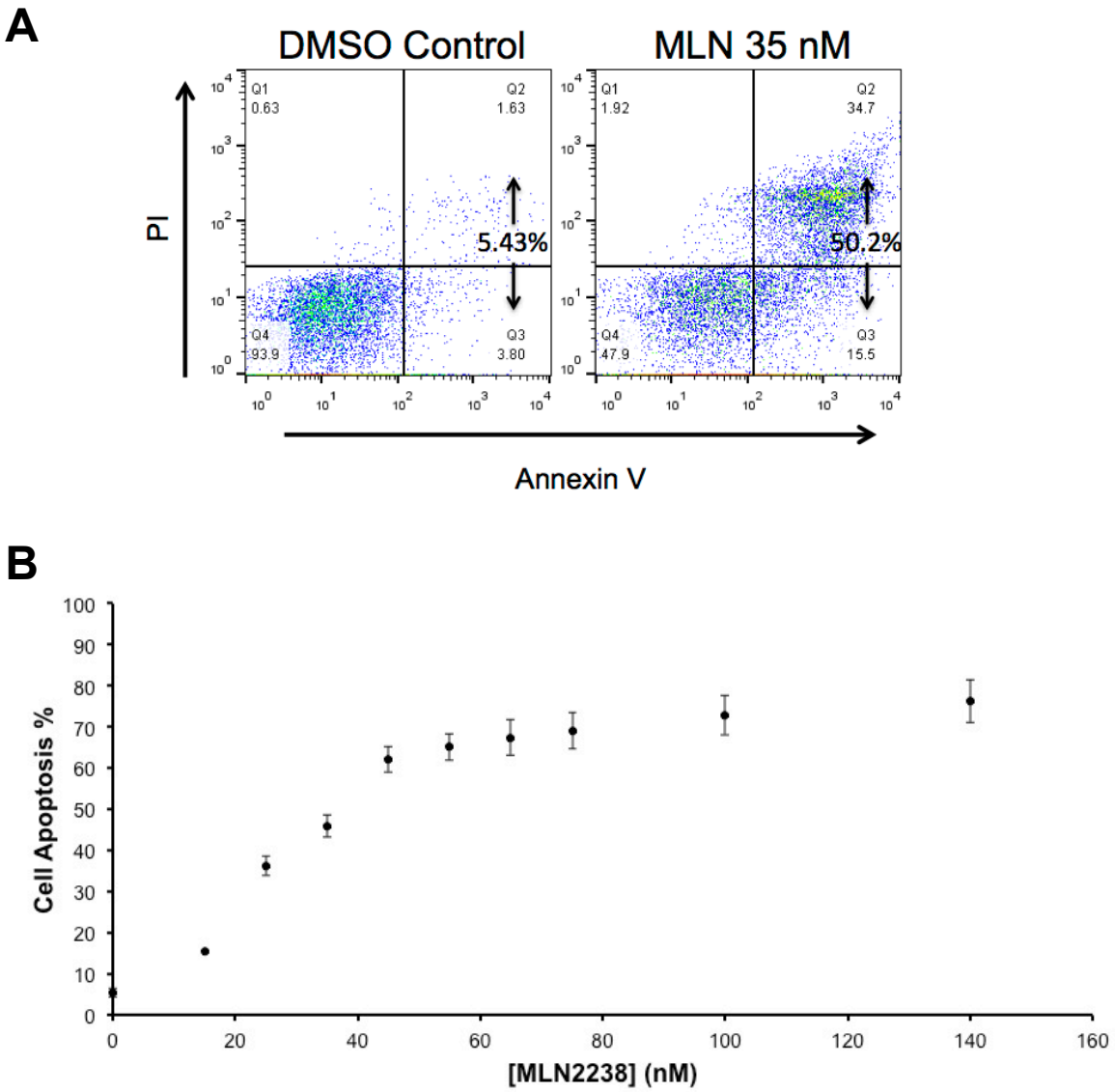


Figure 3. Treatment of BRAF wild type human melanoma cell lines with MLN2238 resulted in apoptotic cell death. A) WM1366 BRAF WT human melanoma cells were plated on a 6 well plate at a density of 7 x 10^4 cells/mL and were treated for 48 hours with either medium alone or with 35 nM MLN2238. After incubation, the cells were subjected to an Annexin V/Propidium Iodide assay to determine the levels of apoptosis. 35 nM MLN2238 treatment resulted in 50.2% apoptotic cells. **B)** A titration was performed with varying doses of MLN2238 to determine the IC_{50} concentration. The MLN2238 IC_{50} value for the WM1366 cell line is approximately 38.4 nM.

Treatment of melanoma cell lines with either bortezomib or MLN2238 resulted in apoptosis. Bortezomib is an antitumor compound that specifically and reversibly inhibits the 26S proteasome. As a single agent, bortezomib has an acceptable toxicity profile and has shown activity in patients with advanced multiple myeloma and mantle cell lymphoma. MLN2238 is a second-generation proteasome inhibitor that exhibits improved pharmacokinetics as an oral agent. These two agents were compared. Human melanoma tumor cells (A375 or WM1366) were treated for 24 or 48 hours with 35 nM MLN2238 or 10 nM bortezomib. These dosages of MLN and bortezomib result in similar levels of cell death (Figure 4). Apoptosis was evaluated using Annexin V/PI staining. 24-hour treatment of the A375 (BRAF mutant) cell line with 35 nM MLN2238 resulted in 16.9% cell death compared to 14.1% cell death observed in cells treated with 10 nM bortezomib. After 48 hours, MLN2238 treatment resulted in 55.4% cell death compared to 50.4% cell death for cells treated with bortezomib (Figure 4A). A similar trend was observed in the BRAF WT (WM1366) human melanoma cell line (Figure 4B).

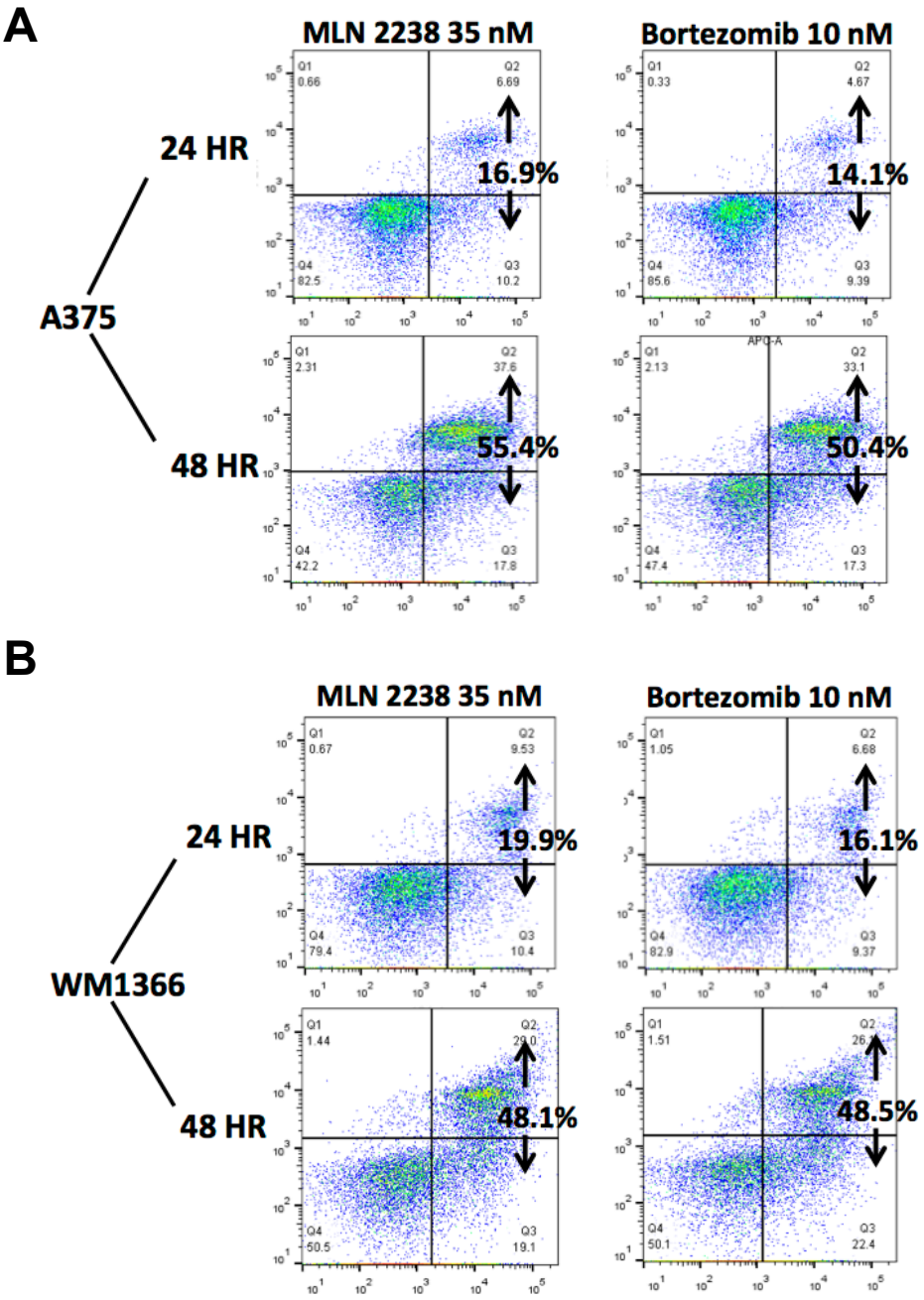


Figure 4. Treatment of melanoma cell lines with MLN2238 and bortezomib results in apoptosis. Mutant BRAF V600E A375 or BRAF WT WM1366 human melanoma tumor cells were treated for 24 or 48 hours with 35 nM MLN2238 or 10 nM bortezomib. Apoptosis was evaluated using Annexin V/PI staining. **A)** Levels of apoptosis in the BRAF mutant (A375) cell line increased from 16.9% at 24 hours to 55.4% at 48 hours from 35 nM MLN2238. Similar results were seen with bortezomib. **B)** Treatment of the BRAF WT (WM1366) cell line with 35 nM MLN2238 resulted in levels of cell death that increased from 19.9% to 48.1% at 24 and 48 hours, respectively. Similar results were seen with bortezomib treatment.

The addition of IFN-alfa to proteasome inhibitor treatment resulted in increased melanoma cell apoptosis. To evaluate the ability of MLN2238 and IFN-alfa combination therapy to induce melanoma cell apoptosis, either the A375 or the WM1366 human melanoma cell lines were treated for 48 hours with IFN-alfa ($5x10^3$ U/mL), MLN2238 (35 nM), bortezomib (10 nM), or the combination of each agent with IFN-alfa. Levels of apoptosis were measured by Annexin V/PI staining. MLN2238 or bortezomib in combination with IFN-alfa led to synergistically increased levels of apoptosis in BRAF mutant melanoma cells as compared with either agent alone (Fig. 5A). For example, combination treatment with MLN2238 and IFN-alfa of A375 cells for 48 hours resulted in 81.7% apoptotic cells compared with 8.24% for IFN-alfa alone and 51.9% for MLN2238 alone. For the WM1366 human melanoma cell line, 35 nM MLN2238 treatment in combination with IFN-alfa resulted in 60.1% cell death compared to 13.3% cell death in cells treated with only IFN-α and 49.2% in cells treated with only 35 nM MLN2238 (Figure 5B).

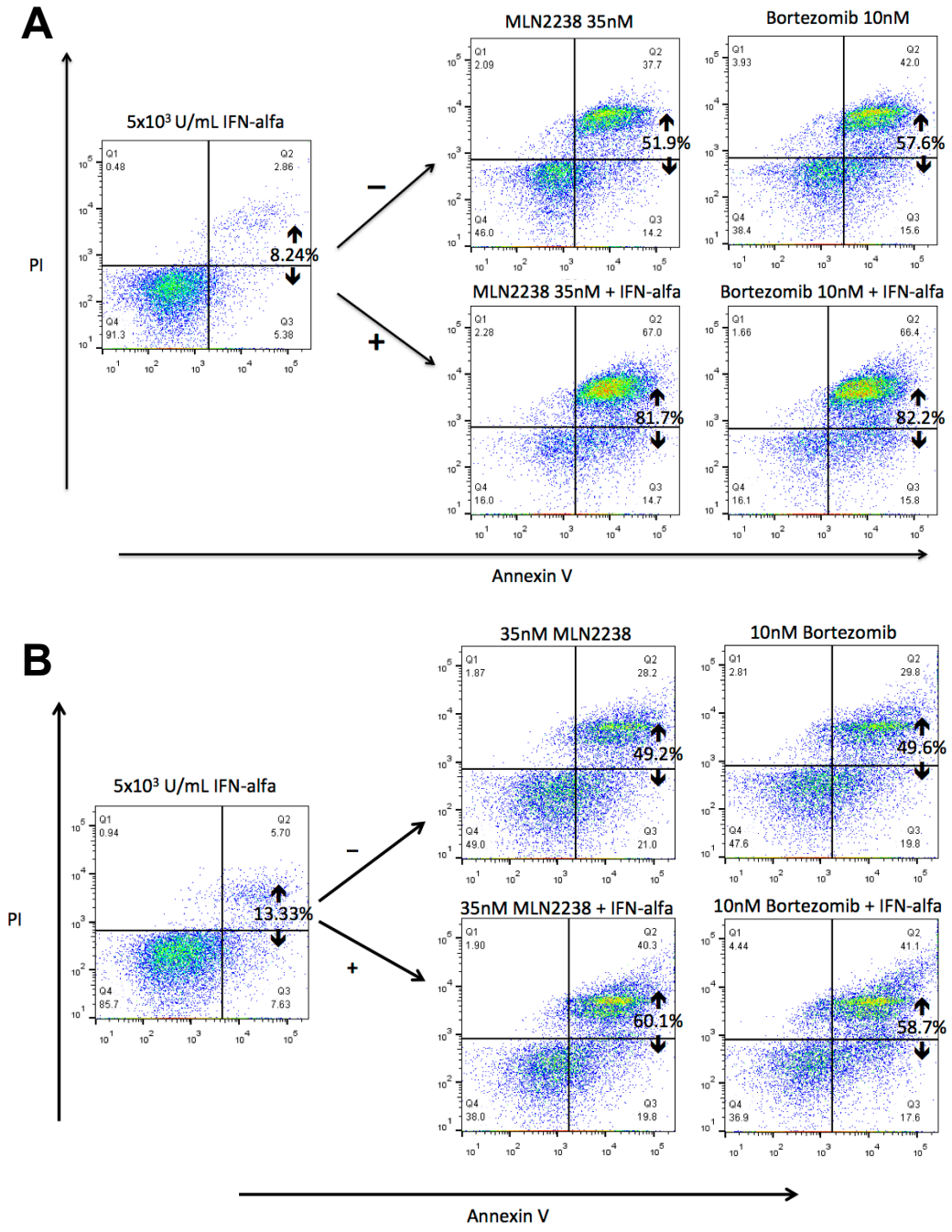
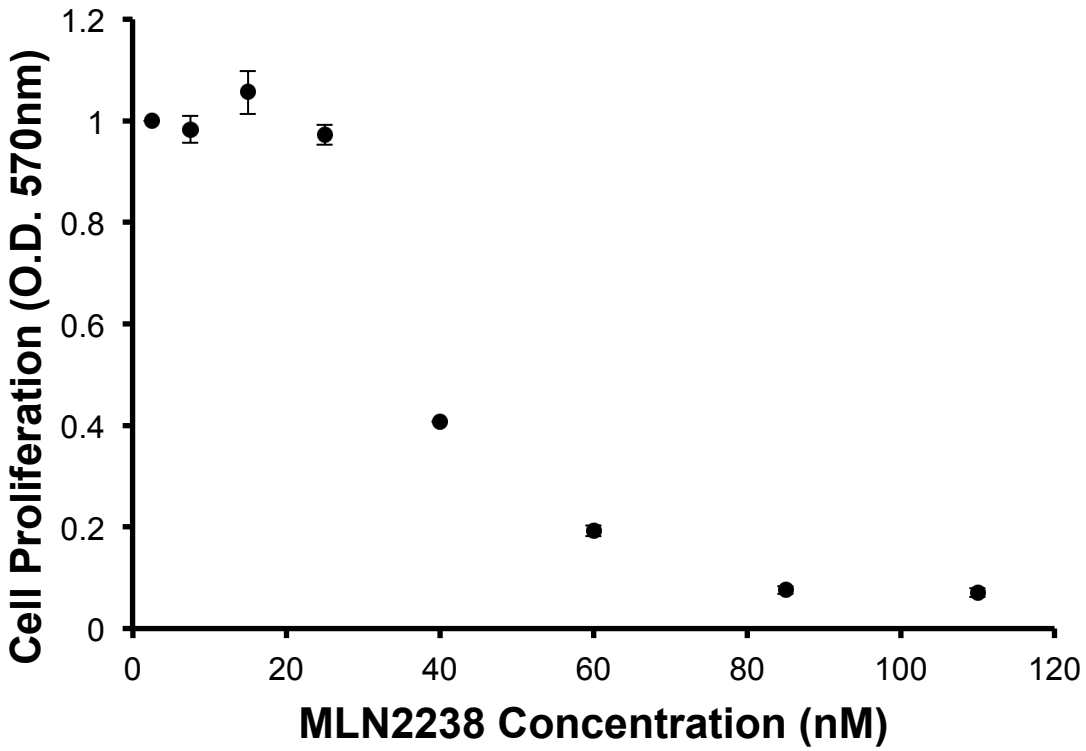


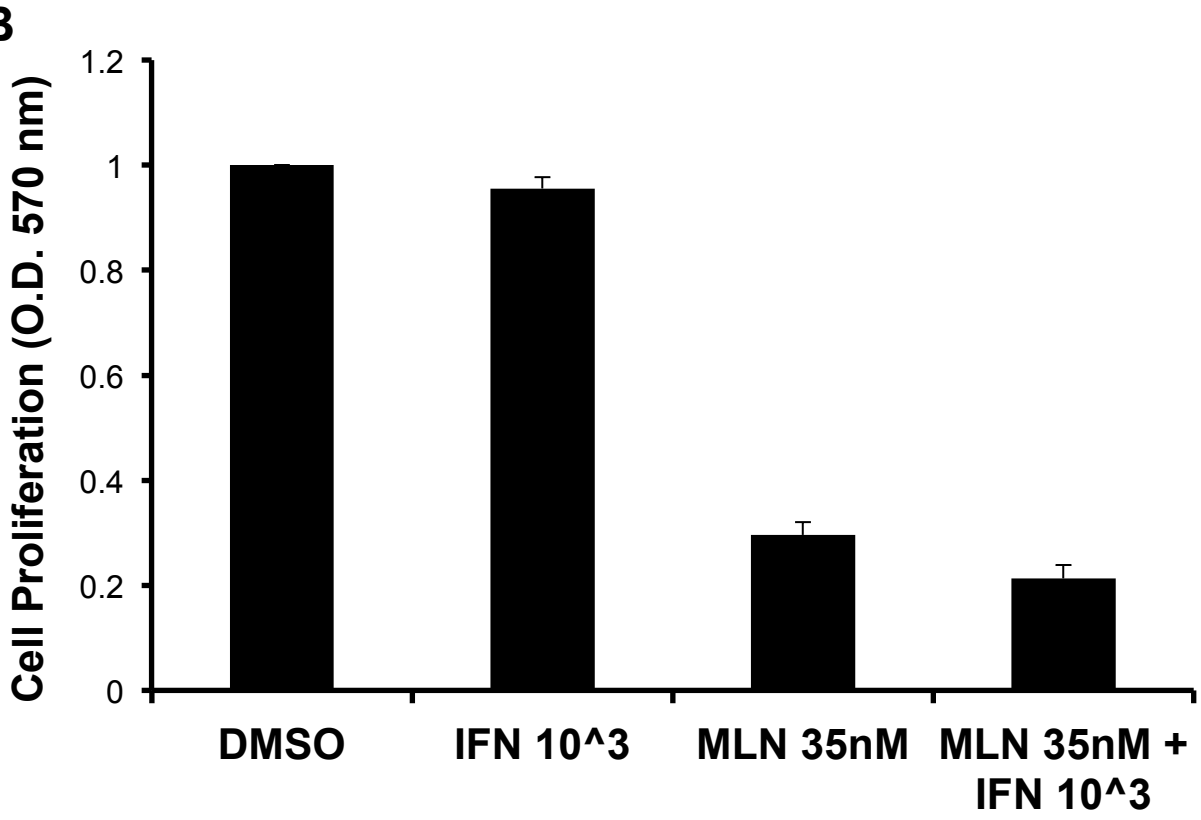
Figure 5. The addition of IFN-alfa to proteasome inhibitor treatment resulted in melanoma cell apoptosis. A) Mutant BRAF V600E A375 human melanoma tumor cells were treated for 48 hours with 35 nM MLN2238 or 10 nM Bortezomib with or without $5x10^3$ Units/mL IFN-alfa. Levels of apoptosis were assessed by Annexin V/PI staining. The addition of IFN-alfa to MLN2238 treatment led to a 57% relative increase in the level of apoptosis while the addition of IFN-alfa to bortezomib led to a 43% relative increase in apoptosis. **B)** BRAF WT WM1366 human melanoma cells were exposed to the same experimental conditions. The addition of IFN-alfa to MLN2238 treatment resulted in a 22% relative increase in apoptosis while the addition of IFN-alfa to bortezomib treatment resulted in an 18% relative increase in apoptosis.

MLN2238 in combination with IFN-alfa resulted in reduced tumor cell proliferation in BRAF mutant human melanoma cells as measured by MTT assays. Cell proliferation was measured as absorbance (O.D.) at 570 nm using a MTT cell proliferation assay. A375 BRAF mutant human melanoma cells were treated with various concentrations of MLN2238 to create a titration curve to evaluate the effect on tumor cell proliferation (Figure 6A). The 35 nM dose of MLN2238 was utilized because of its proximity to the IC_{50} value to allow us to determine synergism and also because it is achievable in humans. BRAF mutant (A375) melanoma cells were then treated with either 35 nM MLN2238, 10^3 U/mL IFN-alfa, or both agents combined for 48 hours (Figure 6B). Cells treated with DMSO alone served as a solvent control. Combination treatment resulted in a larger decrease in cell proliferation than either agent alone. This same procedure was repeated for the WM1366 BRAF WT and NRAS mutant cell line (Figures 6C and 6D). The WM1366 melanoma cell line was more resistant to MLN2238 treatment as measured by the MTT proliferation assay. There was no significant decrease in cell proliferation when compared to the DMSO solvent control. This procedure was again repeated for the MeWo BRAF WT and NRAS WT melanoma cell line (Figures 6E and 6F). The BRAF WT MeWo cell line had no significant level of inhibition of cell proliferation from MLN2238 with or without IFN-alfa treatment. Similar results were observed for bortezomib treatment.

A


Cell Proliferation (O.D. 570nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
MLN2238 Concentration (nM)

B


Cell Proliferation (O.D. 570 nm)
1.2
1
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0.4
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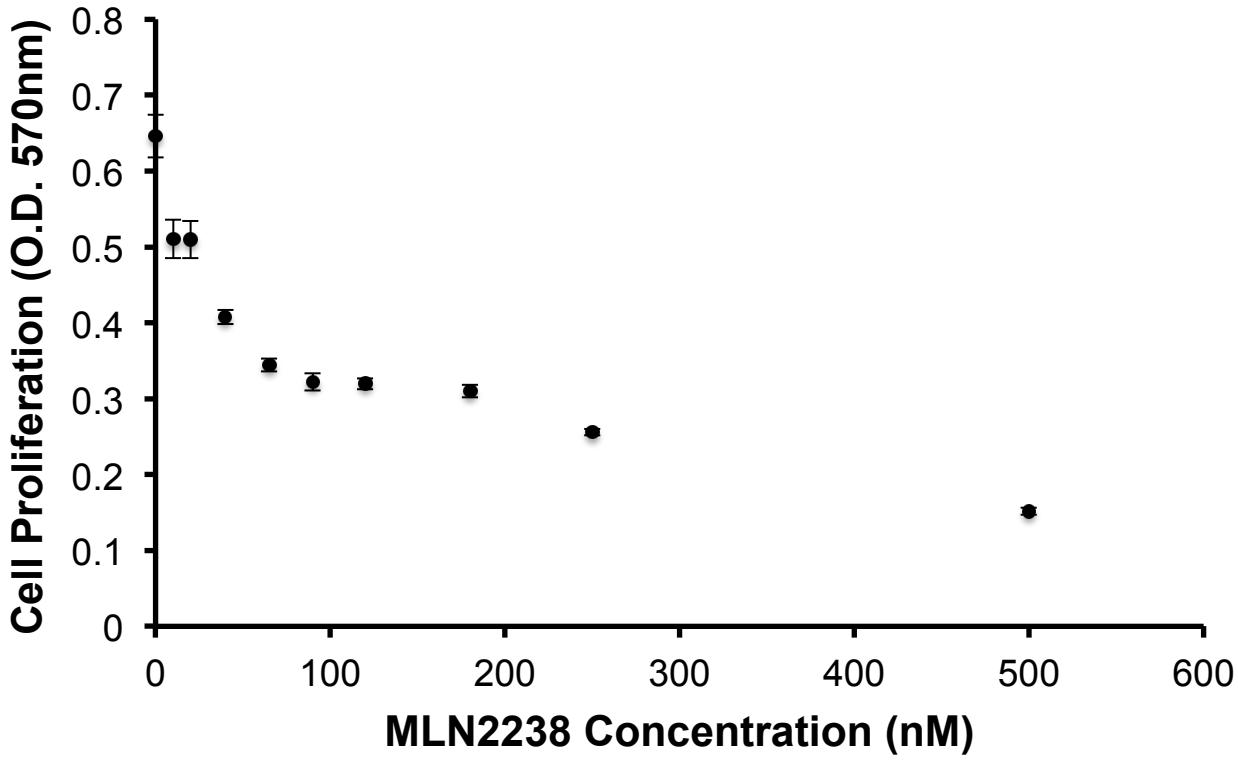
DMSO
IFN 10^3
MLN 35nM
MLN 35nM + IFN 10^3

C


Cell Proliferation (O.D. 570nm)
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0.7
0.6
0.5
0.4
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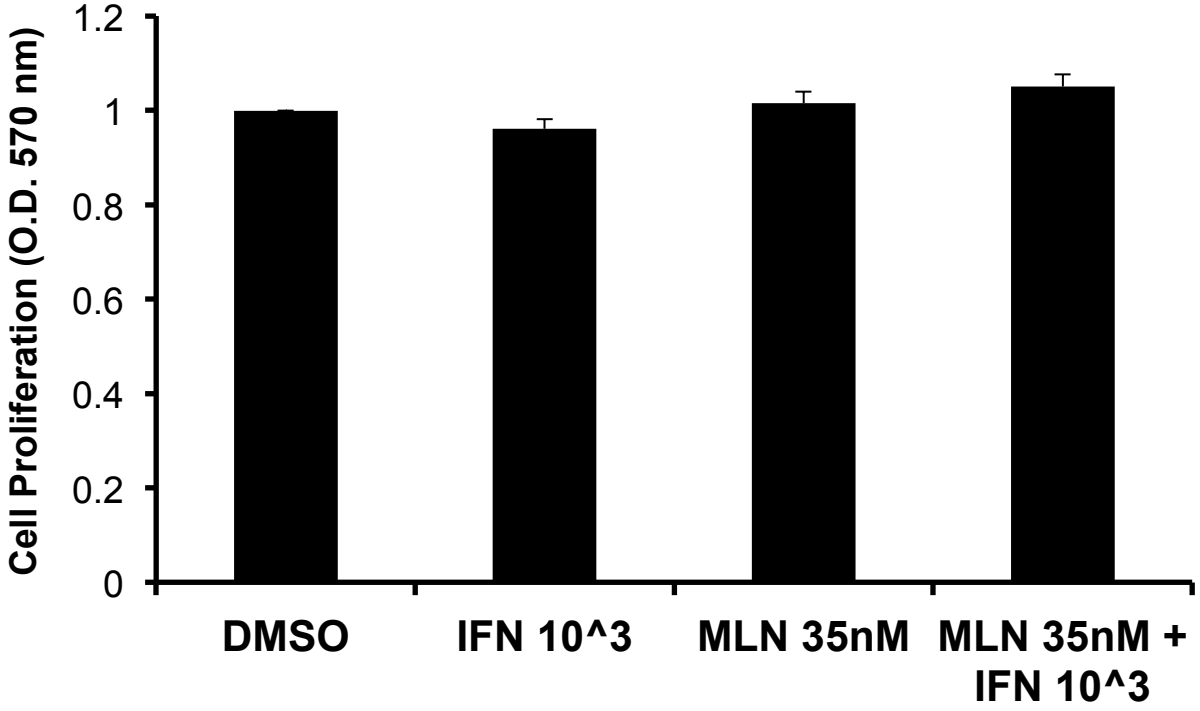
0.1
0
0
100
200
300
400
500
600
MLN2238 Concentration (nM)

D


Cell Proliferation (O.D. 570 nm)
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1
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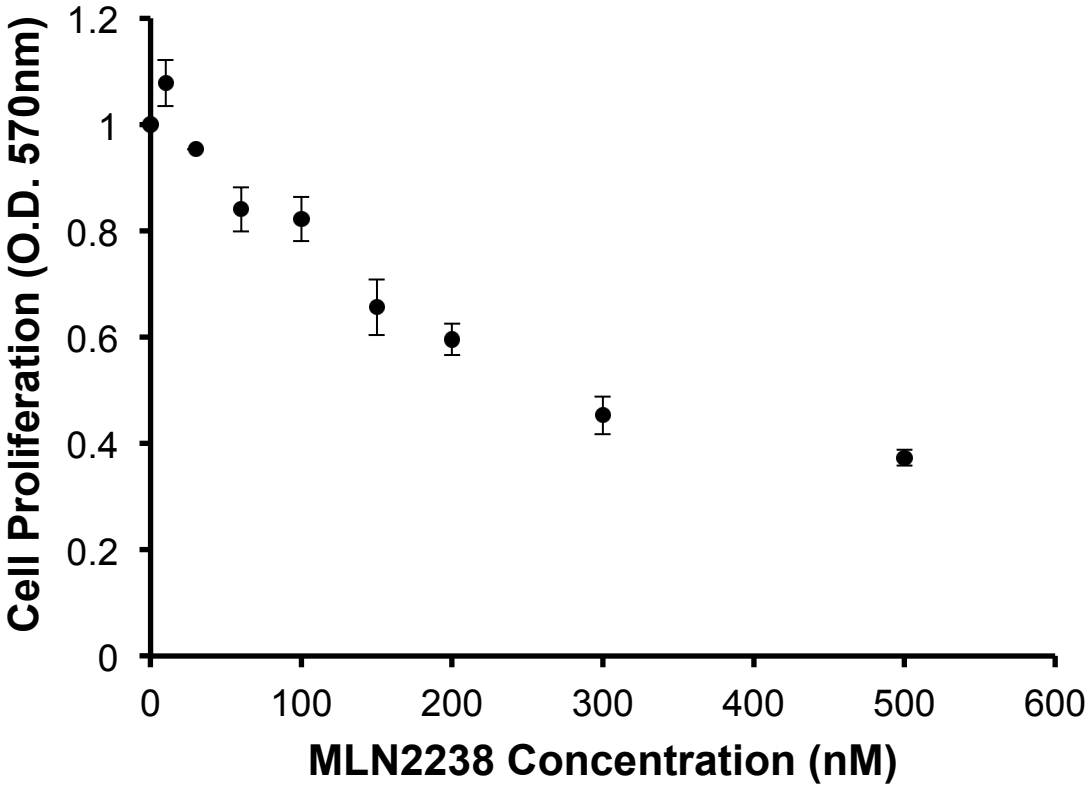
0.6
0.4
0.2
0
DMSO
IFN 10^3
MLN 35nM
MLN 35nM + IFN 10^3

E


Cell Proliferation (O.D. 570nm)
1.2
1
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0.6
0.4
0.2
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0
100
200
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500
600
MLN2238 Concentration (nM)

F

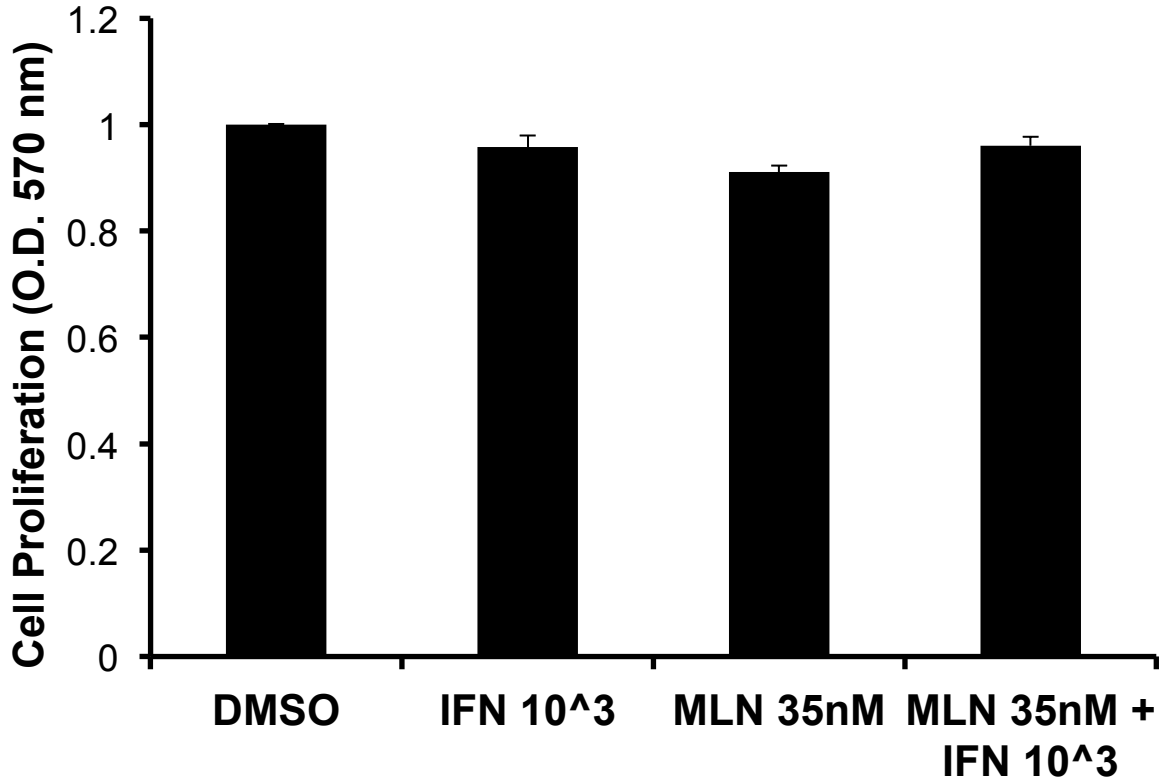

Cell Proliferation (O.D. 570 nm)
1.2
1
0.8
0.6
0.4
0.2
0
DMSO
IFN 10^3
MLN 35nM
MLN 35nM + IFN 10^3

Figure 6. MLN2238 in combination with IFN-alfa resulted in reduced tumor cell proliferation in BRAF mutant human melanoma cells as measured by the MTT assay. A) Human melanoma A375 cells were plated at a density of $3x10^4$ cells/mL in a 96 well plate and treated with various concentrations of MLN2238 for 48 hours. Following incubation, a dye that is a marker for proliferation (MTT) was added followed by the addition of DMSO to dissolve the resulting tetrazolium salt. The 96 well tissue culture plate was then read on a plate reader at 570nm to measure cell proliferation. The IC_{50} value of proliferation was determined to be approximately 35.2 nM. **B)** The same procedure was repeated using 35 nM MLN2238 with or without 10^3 U/mL IFN-alfa for the A375 BRAF mutant cell line. Combination therapy resulted in a statistically significant reduction in tumor cell proliferation compared to either agent alone. **C)** Human melanoma WM1366 cells were plated at a density of $3x10^6$ cells/mL in a 96 well plate and treated with various concentrations of MLN2238 for 48 hours. The IC_{50} value for proliferation was determined to be approximately 90 nM. **D)** The same procedure was repeated using 35 nM MLN2238 with or without 10^3 U/mL IFN-alfa. Combination therapy was not effective. **E)** Human melanoma BRAF WT MeWo cells were plated at a density of $3x10^6$ cells/mL in a 96 well plate and treated with various concentrations of MLN2238 for 48 hours. The IC_{50} value of proliferation was determined to be approximately 260 nM. **(F)** The same procedure was repeated using 35 nM MLN2238 with or without 10^3 U/mL IFN-alfa. Combination therapy was not effective.

MLN2238 therapy induced processing of effector caspases and PARP. Enhanced processing of the major effector caspases (caspase-3, caspase-7, caspase-8 and caspase-9) to their active forms and cleavage of PARP (a target of activated effector caspases) was observed at 48 hours following treatment of the A375 human melanoma cell line with 15-35 nM MLN2238 (Fig. 7).

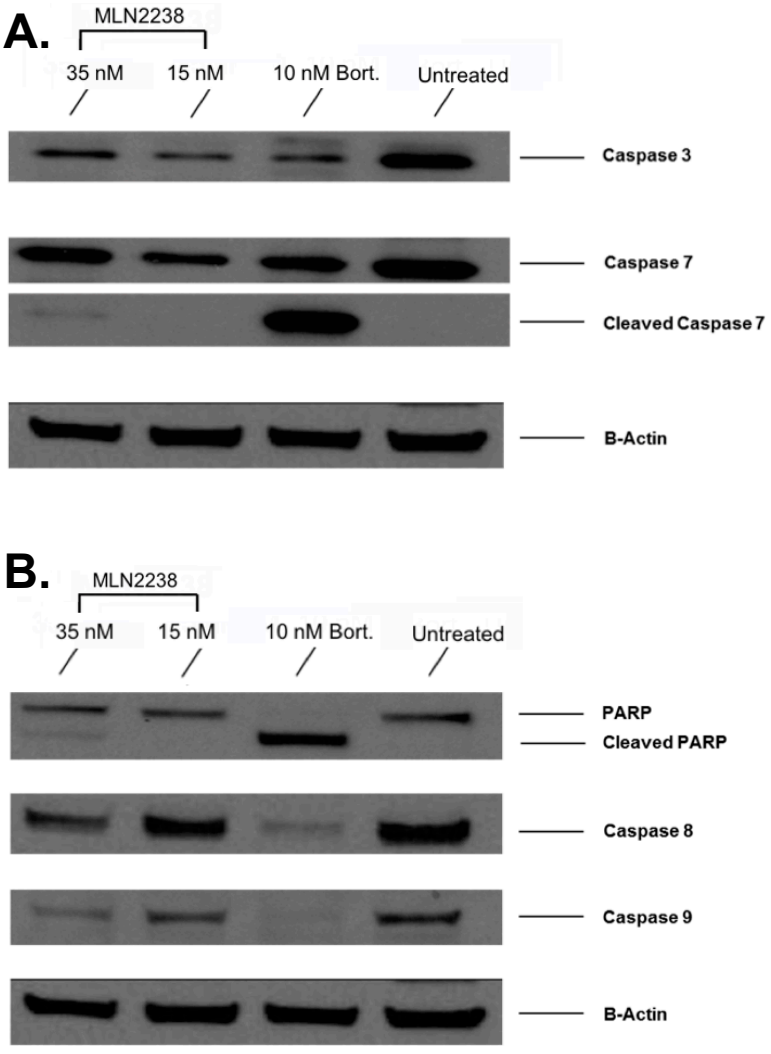


Figure 7: MLN2238 therapy induces processing of apotosis factors such as effector caspases and PARP. A375 melanoma cells were treated with media, 15nM MLN2238, 35nM MLN2238, or 10nM Bortezomib for 48 hours. Treatment of the A375 with MLN2238 resulted in increased levels of processed caspase-3, caspase-7, caspase-8, caspase-9, and PARP, which are key proteins involved in apoptosis.

Discussion

MLN2238 is a second-generation proteasome inhibitor that has improved pharmacodynamics with a six-fold faster proteasome disassociation half-life compared to bortezomib [20]. It is also orally available via a 4.0 mg capsule to help maintain initial serum concentrations and to provide convenience to patients [20]. The aim of this study was to evaluate MLN2238 treatment in conjunction with IFN-alfa as a potential treatment regimen for malignant melanoma. We hypothesized that MLN2238 would induce apoptosis in melanoma cells, which would be increased by the addition of IFN-alfa. Based on our results, MLN2238 treatment did induce apoptosis in both BRAF wild type and BRAF mutant melanoma cell lines but had increased efficacy in a BRAF mutant melanoma cell line (A375).

MLN2238 in combination with IFN-alfa resulted in an apparent synergistic increase in the levels of apoptosis for BRAF mutant cell lines (A375). MLN2238 induced apoptosis in BRAF WT melanoma cell lines (WM1366). However, there was increased resistance to MLN2238 treatment in the WM1366 cell line. The effects of MLN2238 treatment in combination with IFN-alfa were decreased as compared to the A375 cell line.

A synergistic decrease in cell proliferation was observed with the combination of IFN-alfa and 35 nM MLN2238 in BRAF mutant (A375) melanoma cells. However, the 35 nM dose of MLN2238 had no effect on BRAF WT (WM1366 and MeWo) melanoma cell proliferation as measured by the MTT dye that quantifies metabolic activity. The addition of IFN-alfa had negligible effects on the BRAF WT cell lines. As was seen in Annexin-PI apoptosis assays, MLN2238 had more activity in the BRAF mutant cell line.

MLN2238-induced apoptosis was associated with the processing of procaspase-3, procaspase-7, procaspase-8, procaspase-9, and cleavage of PARP. It may be that MLN2238 induces apoptosis through FADD-induced caspase-8 activation, as was seen in previous studies performed by Dr. Carson's group [10].

In addition to our studies, other groups have displayed synergistic relationships involving IFN-alfa with various other drugs in several different cancer models. One such study examined sorafenib, an oral multikinase inhibitor, in combination with pegylated IFN-alfa-2b on human liver cancer cells. The combination of these two drugs resulted in synergistic induction of cell death *in-vitro*, and the reduction of tumor volume in a murine *in-vivo* model [21]. Another study examined a potent selective inhibitor of Bcl-2 (ABT-263) in combination with MLN2238 in prostate cancer. Each drug displayed mild cytotoxicity in single agent use, but when used in dual treatment they displayed a synergistic relationship in prostate cancer cells [22].

In a recent study examining the BRAF V600E mutation, it was determined that proteasome inhibitors only had a significant effect on BRAF mutant colorectal cell lines [23]. When BRAF V600E inhibitors were added in combination with proteasome inhibitors, the effects from the proteasome inhibitor treatment were negated [23]. This suggests that the BRAF V600E gene needs to be constitutively expressed for proteasome inhibitors to be active; otherwise resistance to proteasome inhibitors develops. This is likely responsible for the differences in MLN2238 sensitivity between the BRAF mutant and BRAF wild type melanoma cell lines used in this study. It also could be a factor in the results from the phase I bortezomib clinical trial conducted since BRAF status was not taken into account [13].

A recent study examined 21 different compounds and their potency on three different canine melanoma cell lines. Bortezomib displayed the most significant decreases in cell growth in the *in-vitro* setting [24]. The researchers further investigated bortezomib in an *in-vivo* setting using a CMM-1 melanoma xenograft murine model. Bortezomib treatment resulted in statistically significant levels of tumor growth suppression, and also resulted in a significant decrease in mitotic index when compared to control tumors [24].

The Carson group has also seen decreases in tumor growth with bortezomib in both in vitro and in murine model settings. It is possible that improved pharmacokinetics of MLN2238 will result in improved outcomes for patients. MLN2238 has a shorter proteasome dissociation half-life compared to bortezomib (18 vs. 110 minutes), along with a larger blood volume distribution at a steady rate [20,25]. With a shorter proteasome dissociation half-life, MLN2238 is capable of achieving greater and more constant biodistribution than bortezomib [20]. This suggests that MLN2238 may have increased efficacy in combination with IFN-alfa in the clinical setting due to more constant serum concentrations.

The results from this study and additional supporting studies indicate potential for a novel melanoma treatment regimen. IFN-alfa has the capacity to synergistically act with MLN2238 to selectively increase BRAF mutant melanoma tumor cell apoptosis and should be further investigated in a clinical setting.

Appendices:

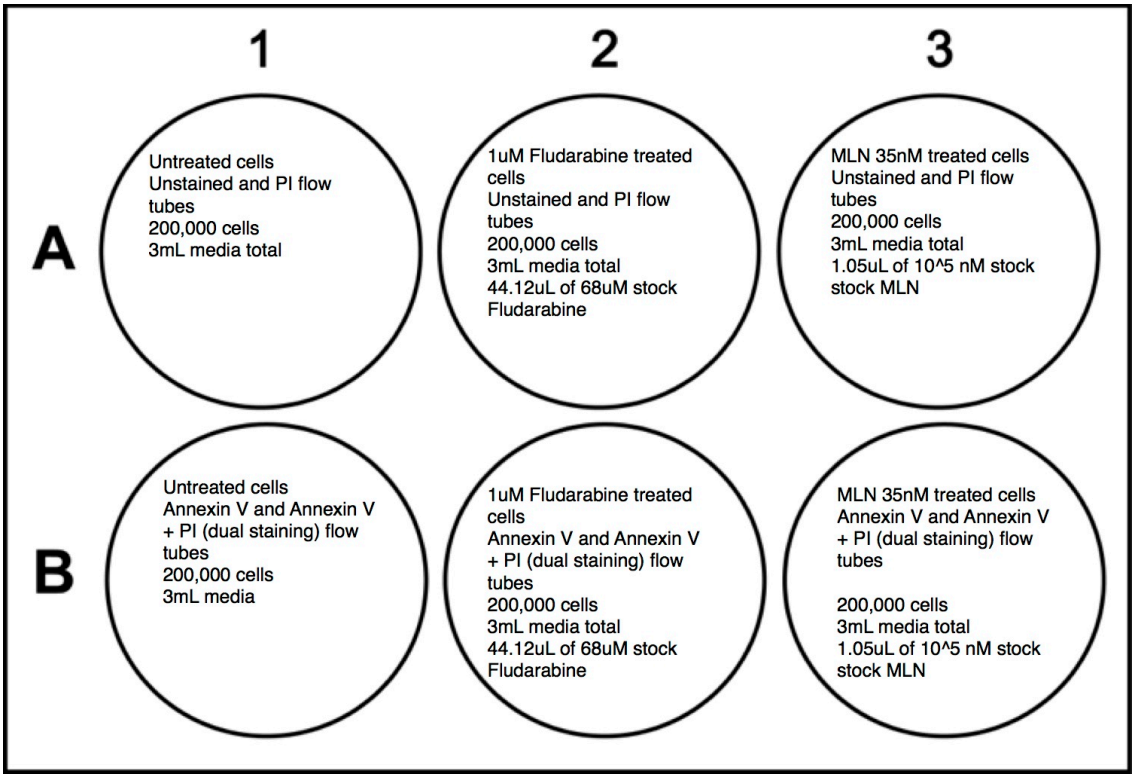
Appendix A

Annexin/PI Protocol:

1. Thaw cells from freezebacks in liquid nitrogen. Place freezeback in 37 °C water bath for approximately 3-4 minutes.
2. Transfer cells to a 50 mL conical tube and add 10 mL of media using a 1 mL serological pipette.
3. Centrifuge the cells at 1700 RPM for 10 minutes.
4. Resuspend pellet in 1mL of complete media, and count cells using a 5uL aliquot into 95 uL of trypan blue solution.
5. Count the cells and then resuspend them at 10^6 cells/mL.
6. Prepare and label your 6 well plates (Cellstar Product #657160). You do not want to add more than 200,000 cells per well, and want 100,000 cells per flow tube. For your positive and negative controls, you want to have an unstained flow tube, PI only stained flow tube, Annexin V only stained flow tube, and a Annexin V and PI stained flow tube. You also want to have these four flow tubes for each stimulus you have in order to be able to set your quadrants. For example, if you will have 4 flow tubes for a sample, you will have to have 2 wells for that condition and will then combine the cells after incubation.
7. Add 200,000 cells (200uL) per well in a 6 well plate.
8. Then add 2.8 mL of media to each well to reach a final volume of 3mL
9. Thaw each agent you will be using at room temperature. Keep at room temperature for as little time as possible.

10. Treat cells with stimulus.

11. Go through each well and pipette up and down gently without scraping the well with a 1000uL tip to mix each solution. Below is an example of a 6 well plate layout.



12. Incubate the well plates at 37 °C for desired time point

13. After incubation, remove the contents of each well using a serological pipette into their own respective labeled 15 mL conical tube. Try to avoid scratching the bottom of the well. Each condition you have should have one 15mL conical tube. If you have a condition that has 2 separate wells (4 flow tubes), combine the contents of each well into the same 15 mL conical tube.

14. Add 3mL of 0.25% trypsin-EDTA to each well and incubate at 37 °C for 4 minutes
15. Add 2 mL of media to each well to neutralize it
16. Collect the 5 mL from each well and add that to their same respective 15mL conical tube as in step 8
17. Centrifuge the 15 mL conical tubes for 10 minutes at 1700 rpm
18. Very slowly aspirate each tube by using a glass pipette. Keep the glass pipette as far from the pellet as possible, while slowly tilting the conical tube until the media is aspirated.
19. Prepare 1X Annexin V Binding buffer by diluting the stock Annexin V 10X Binding Buffer with DEPC H2O. Usually a 10mL aliquot will be prepared by adding 1 mL of Annexin V 10X binding buffer with 9ml of DEPC H2O. Adjust the aliquot size for the amount of samples you have.
20. Resuspend the pellets in the 15 mL conical tubes in 1x Annexin V Binding Buffer. If the condition will be having 4 flow tubes (and had 400,000 cells), resuspend the pellet in 800uL of 1x Annexin V Binding Buffer. If the condition will be have 2 flow tubes (and had 200,000 original cells), resuspend in 400uL of 1x Annexin V binding buffer.
21. Transfer 200uL of each suspension to their respective flow tubes.
22. DONE IN DARK - Then add 5 μL of anti-Annexin V- APC conjugated Antibody to each flow tube and/or 10 μL of PI as appropriate for each flow tube.
23. Once the appropriate stains are added to each flow tube, incubate the flow tubes on ice for 15 minutes. The samples should remain on ice for the

remainder of the experiment. Also place the remaining 1X Annexin V binding buffer on ice for the next step.

24. After the 15-minute incubation, add another 200 μL of the cold 1X Annexin V binding buffer to every flow tube.
25. **The samples NEED to be immediately run on the LSR flow cytometer**. If you wait more than 15-20 minutes after you complete the staining you WILL see a noticeable difference in your results and will mostly have to repeat the experiment.
26. When running your samples on the LSR, first use your untreated and unstained cells to find your cells. Run the LSR in exponential mode. You want the Y-axis to be PE and the X-axis to be APC. You want to collect 10,000 events for your controls, and 30,000 events for your experiment samples. You want to position the cells as close to the lower left corner as possible without cutting off populations. Then go through your dual stained positive control to make sure everything looks good.
27. Analyze the results using FlowJo. Use your PI only and Annexin V only controls to set your quadrants. You can then see the amounts of apoptosis (top and bottom right hand quadrants).

Product	**Company**	**Product Number**
APC Annexin V	Fisher	550475
Propidium Iodide	Fisher	556463
Annexin V Binding Buffer	Fisher	556454
6 Well Plates	USA Scientific	T1006

Appendix B:

MTT Cell Proliferation Assay Protocol

Preliminary cell count assay:

1. First a cell titration must be performed to determine the proper amount of cells needed to obtain an absorbance reading inside the linear range of the plate reader (This was 0.8-1.2 at the time of this protocol. This may change with improving technology).
2. Perform serial dilutions for each cell concentration. Start with a concentration of $2x10^6$ cells/mL and continue to $5x10^3$ cells/mL (if using cancer cells). Prepare the concentrations so that the total volume in each well is 100 µL. Follow the remainder of the protocol to complete this preliminary assay.

MTT Assay:

3. Thaw cells of choice from liquid nitrogen in a 37 degrees water bath.
4. Centrifuge cells at 1700 RPM for 10 minutes.
5. Remove the supernatant via a glass pipette and resuspend in 5mL of its respective media.
6. Add the suspension to 15 mL more of media and grow out the cells in a T75 flask until confluent.
7. When cells are confluent, aspirate the T75 flask and add 4mL of 0.25% Trypsin for approximately 4 minutes.
8. Immediately add 6mL of media to neutralize the trypsin (needs to be greater than 1:1 media to trypsin).
9. Remove the suspension and place in a 50 mL conical tube.

10. Centrifuge for 10 minutes at 1700 RPM.
11. Remove the supernatant via a glass pipette and resuspend in 1mL of media via a 1000 μL pipette.
12. Add 5uL of the cell suspension into 95uL of Trypan Blue and count the cells.
13. Resuspend the cells so that the concentration is 10^6 cells/mL.
14. Prepare each treatment condition in 1.5 mL Eppendorf tubes.
15. Calculate your dilutions so that the final volume is 1 mL in each tube.
16. The cell number selected should give an absorbance reading between 0.8-1.5.

    Ex.) 15uL MLN2238 + 30uL of A375 cells + 955uL of A375 media

17. First add the media to each eppendorf tube followed by the cells and then each drug.
18. After each tube is prepared, label a 96 Well Flat Bottom Plate. Make sure each of your treatments is at least done in triplicate. An example of a well plate layout is below.

	1	2	3	4	5	6
A	A375 media	A375 media	A375 media	A375 9nM Bort. + IFN 10^3	A375 9nM Bort. + IFN 10^3	A375 9nM Bort. + IFN 10^3
B	A375 untreated	A375 untreated	A375 untreated	A375 9nM Bort. + IFN 10^4	A375 9nM Bort. + IFN 10^4	A375 9nM Bort. + IFN 10^4
C	A375 IFN 10^3	A375 IFN 10^3	A375 IFN 10^3	WM1366 media	WM1366 media	WM1366 media
D	A375 IFN 10^4	A375 IFN 10^4	A375 IFN 10^4	WM1366 Untreated	WM1366 Untreated	WM1366 Untreated
E	A375 7nM Bort.	A375 7nM Bort.	A375 7nM Bort.	WM1366 IFN 10^3	WM1366 IFN 10^3	WM1366 IFN 10^3
F	A375 7nM Bort. + IFN 10^3	A375 7nM Bort. + IFN 10^3	A375 7nM Bort. + IFN 10^3	WM1366 IFN 10^4	WM1366 IFN 10^4	WM1366 IFN 10^4
G	A375 7nM Bort. + IFN 10^4	A375 7nM Bort. + IFN 10^4	A375 7nM Bort. + IFN 10^4	WM1366 7nM Bort.	WM1366 7nM Bort.	WM1366 7nM Bort.
H	A375 9nM Bort.	A375 9nM Bort.	A375 9nM Bort.	WM1366 7nM Bort. + IFN 10^3	WM1366 7nM Bort. + IFN 10^3	WM1366 7nM Bort. + IFN 10^3

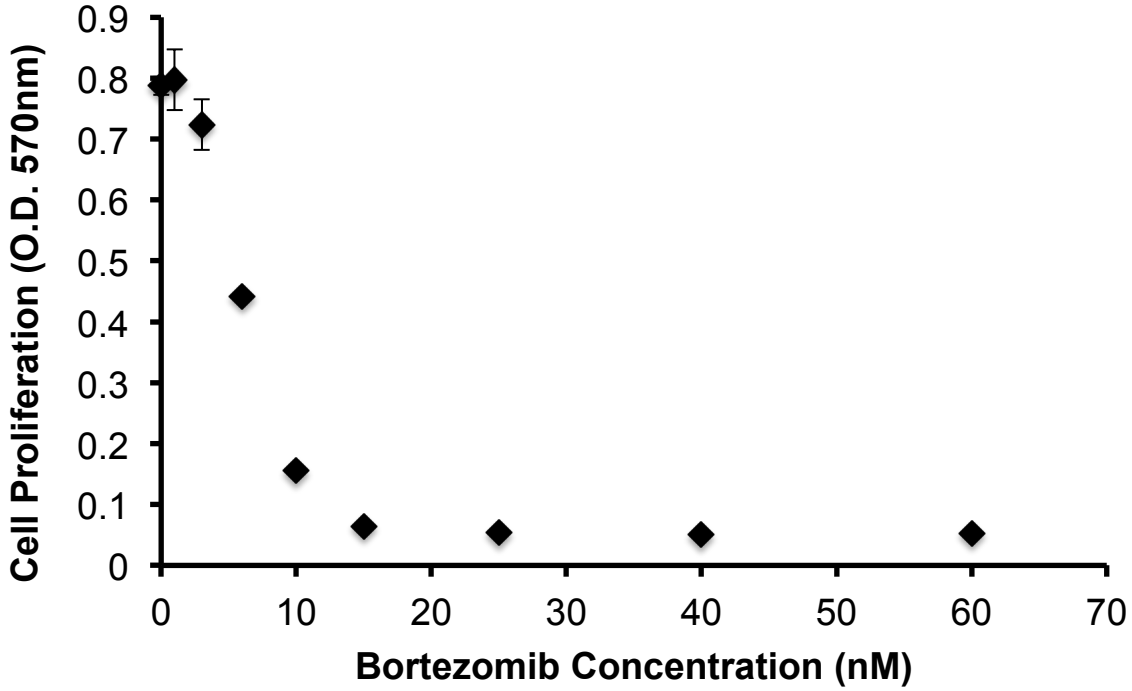
19. Row A should be solely filled with 200 μL of PBS. The rest of the wells should be filled with each of your samples.
20. Then for each sample, add 100 μL of the solution prepared in the eppendorf tube. This should be repeated at least 3 times (if doing triplicate). A 100 μL pipette should be used a new tip should be used for each well.
21. After each treatment is plated, fill the remaining wells (if any) with 200 μL of PBS.
22. Incubate the plate at 37 °C for 48 hours.
23. After the 48-hour incubation, add 5.26 μL of 5.0 mg/mL MTT to each well (except PBS wells). **Everything involving MTT should be done in the dark.**
24. Incubate the well plate at 37 °C for 3 hours.
25. After incubation, remove the contents of each well using a multi channel pipette. Set the volume of the multichannel to slightly over the volume in each well. Make sure the pipette is calibrated.
26. SLOWLY remove the contents of the well plate. Tilt the well plate and gently put the pipette tips at the bottom of the well. Slowly pull up the volume of each well and discard.
27. Then add 100 μL of DMSO using a multichannel pipette to each well.
28. Afterwards, rotate the well plate in your hand in a circular motion fast but not fast enough to spill the contents of each well.
29. Run the plate on the plate reader at 570nm.
30. If there are large error bars for the measurements the odds are you did not mix the solution well enough. Rotate the plate once again and re-read on the plate reader at 570 nm.

Product	Company	Product Number
100 mg MTT	Sigma Aldrich	M5655-100MG
96 Well Flat Bottom Plate	BioExpress	12-565-226

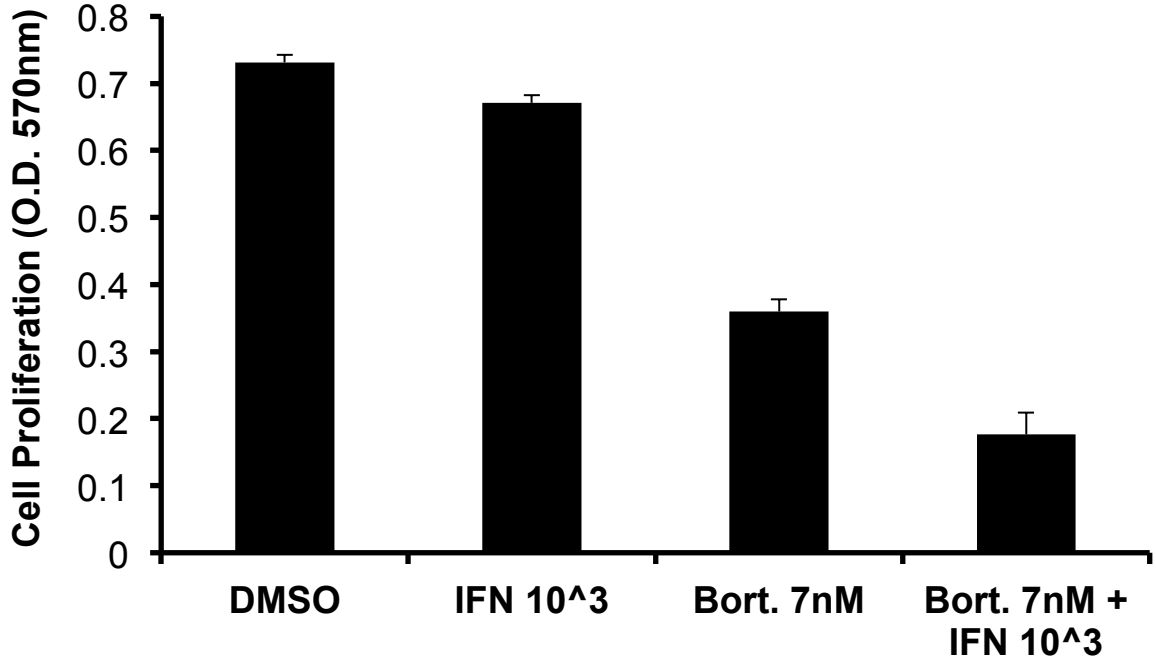
Appendix C

Bortezomib MTT Cell Proliferation Data:

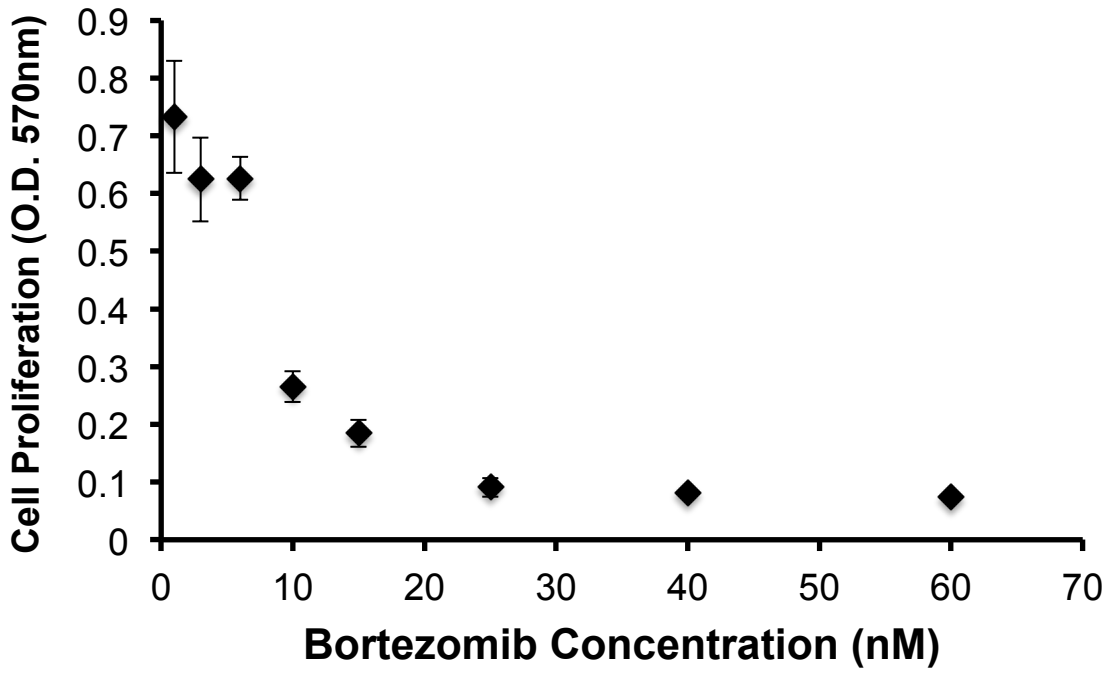
A



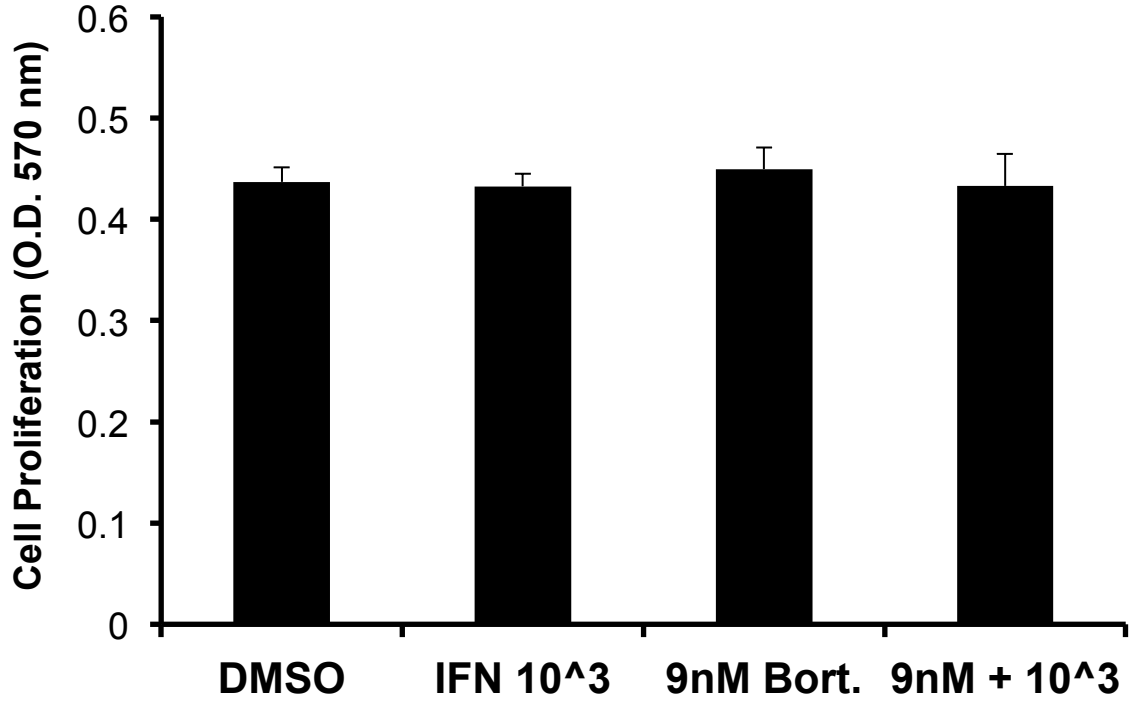
B



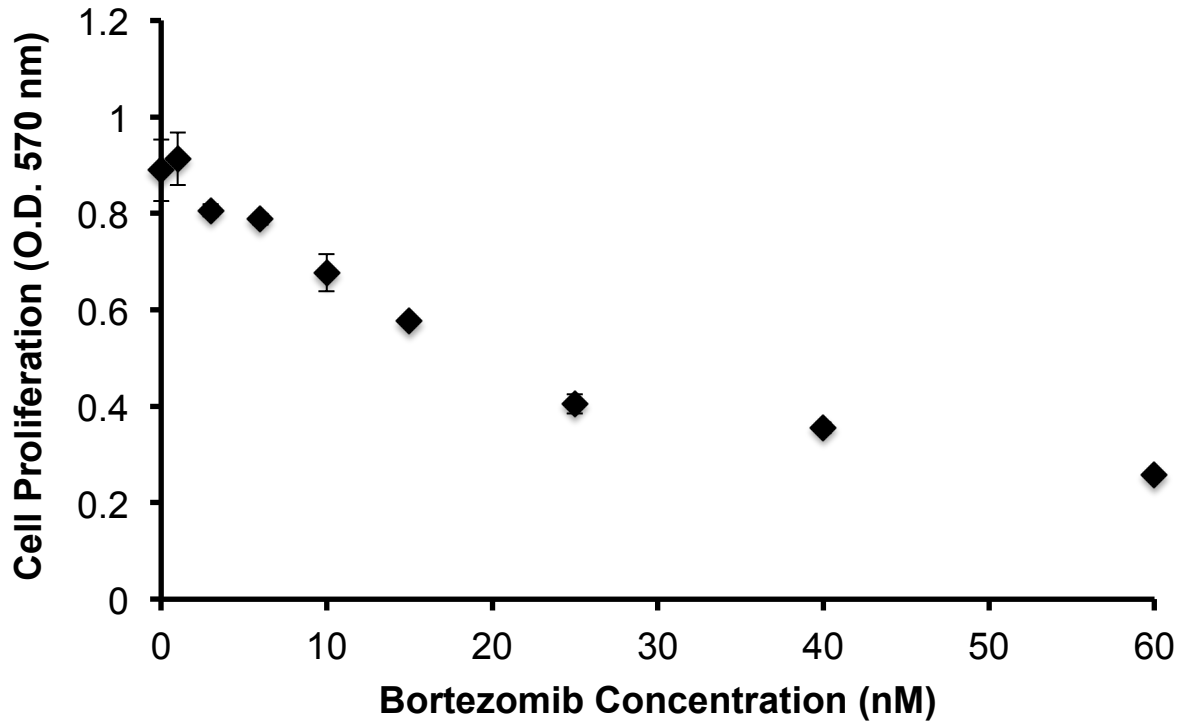
C


Cell Proliferation (O.D. 570nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
Bortezomib Concentration (nM)

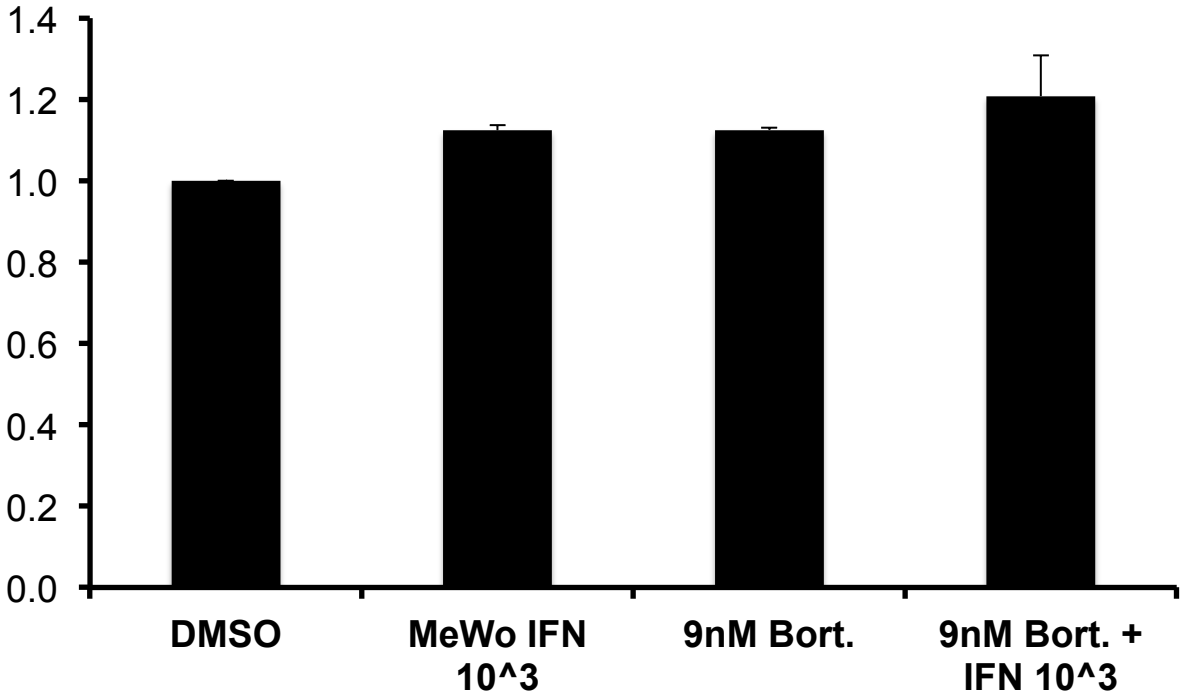
D


Cell Proliferation (O.D. 570 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
DMSO
IFN 10^3
9nM Bort.
9nM + 10^3

E


Cell Proliferation (O.D. 570 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
Bortezomib Concentration (nM)

F


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
DMSO
MeWo IFN 10^3
9nM Bort.
9nM Bort. + IFN 10^3

Appendix C: Bortezomib in combination with IFN-alfa resulted in reduced tumor cell proliferation in BRAF mutant human melanoma cells as measured by the MTT assay. A) Human melanoma A375 cells were plated at a density of $3x10^4$ cells/mL in a 96 well plate and treated with various concentrations of bortezomib for 48 hours. Following incubation, a dye that is a marker for proliferation (MTT) was added followed by the addition of DMSO to dissolve the resulting tetrazolium salt. The 96 well tissue culture plate was then read on a plate reader at 570nm to measure cell proliferation. The IC_{50} value of proliferation was determined to be approximately 7.2 nM. **B)** The same procedure was repeated using 7 nM bortezomib with or without 10^3 U/mL IFN-alfa for the A375 BRAF mutant cell line. Combination therapy resulted in a significant reduction in tumor cell proliferation compared to either agent alone. **C)** Human melanoma WM1366 cells were plated at a density of $3x10^6$ cells/mL in a 96 well plate and treated with various concentrations of bortezomib for 48 hours. The IC_{50} value for proliferation was determined to be approximately 7.3 nM. **D)** The same procedure was repeated using 9 nM bortezomib with or without 10^3 U/mL IFN-alfa. Combination therapy was not effective. **E)** Human melanoma BRAF WT MeWo cells were plated at a density of $3x10^6$ cells/mL in a 96 well plate and treated with various concentrations of bortezomib for 48 hours. The IC_{50} value of proliferation was determined to be approximately 21.2 nM. **(F)** The same procedure was repeated using 9 nM bortezomib with or without 10^3 U/mL IFN-alfa. Combination therapy was not effective.

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